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Biosolids recycling impact on biofilm extracellular enzyme activity and performance of hybrid rotating biological reactors

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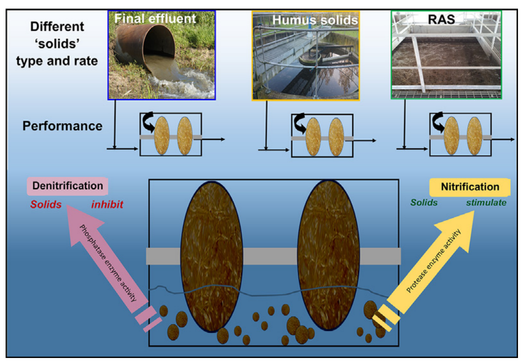
HIGHLIGHTS

- Solids augmentation improved maximum bulks organics and NH_4-N removal ~six fold.
- Final effluent feed had the maximum nitrogen removal rate 71 $g{\cdot}NOx\text{-}N{\cdot}m^{-2}d^{-1}$.
- Amino-peptidase extracellular enzyme activity increased with organic load to 122 $\mu M{\cdot}gVS{\cdot}min^{-1}$.
- Organic load, solids load and type were important operating parameters governing EEA and performance.
- Recycling active solids permitted high EEA despite overloading which improved performance.

GRAPHICAL ABSTRACT



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ABSTRACT

Biological processes for wastewater treatment is limited by extracellular enzyme activity (EEA) of the biofilm on polymeric substrates. The efficiency of biodegradation / biosorption mechanisms causing EEA and organic load removal in biofilms remains unknown. Our hypothesis was that the limiting step of biological process can be overcome by biostimulation and/or bioaugmentation of the return sludge in hybrid biofilm reactors, which leads to competition between suspended and attached bacteria and lower effective substrate to microrganism ratio. Therefore, we considered more active biosolids to perform best at enhancing reactor removal rate. To test this, the efficacy of recycling distinct bio-solids types considered to have different bacterial activity such as final effluent (FE), humus solids (HS) and recycle activated sludge (RAS) on performance improvements of rotating biofilm reactors (RBRs). These bio-solids were investigated under high organic loading rates (OLR) and solids loading rates (SLR) using pilot scale reactors receiving real municipal wastewaters. Controlled overloading of RBRs revealed that EEA improved with increasing OLR/SLR. High SLR (>3.3 kg Total Suspended Solids m^{-2} d^{-1}) delayed and decreased the reduction of organic and inorganic removal rates in the biological processes which commonly occurs under high OLRs. This effect was more pronounced in the highest activity solids (RAS > HS > FE) suggesting the activity and function of bio-solids was critical to improve performance of RBRs. High OLR and SLR induced efficient denitrification and organics removal within the biofilm reactor at residence times of <5 min. Recycling active solids permitted EEA despite overloading which was critical to the performance of the RBRs.

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1. Introduction

Extracellular enzyme activity (EEA) is needed to treat organic wastewater polymers as the majority are too large to be transported directly into bacteria, this is the case for biological processes containing floc forming activated sludge bacteria, granular sludge bacteria and biofilms (Burgess and Pletschke, 2008; Hassard et al., 2018). The degradation of polymeric organic matter in wastewater treatment is a complex step wise process (Hassard et al., 2018). Firstly, polymers are transported through the water, second, the polymeric material is adsorbed to the biofilm, third the polymeric material undergoes a series of stepwise depolymerisation reactions and finally components are assimilated, stored or released by the bacteria. These processes can be slow and therefore, have the potential to restrict the substrate removal rates which can be achieved in wastewater treatment. Of these processes, the extracellular breakdown of organic polymers is thought to be the limiting step restricting the organic loading rate (OLR) which can be applied whilst achieving desired effluent quality (Orhon and Çokgör, 1997; Martins et al., 2003; de Kreuk et al., 2010). Biological processes designed with process conditions of OLRs such as 'roughing' processes are particularly susceptible to EEA limitation (Wingender, 2002; Hassard et al., 2015). Most of the EEA is associated directly with bacterial cell walls or localised within solids matrices such as flocs or granules (Confer and Logan, 1998; Morgenroth et al., 2002). Therefore, biofilm processes have a genuine advantage over traditional flocculated systems through intensification of bacteria and their extracellular enzymes, but a biofilm will usually have a larger barrier to substrate diffusion than a smaller floc. This is pertinent considering that wastewater bacteria have been shown to regulate EEA based on available substrates, electron acceptor conditions and their specific microbial growth rate (Li and Chróst, 2006; Hauduc et al., 2013; Shackle et al., 2000). Thus, wastewater biofilms appear to increase their specific EEA to account for the increased barrier to diffusion. This suggests, therefore, that EEA could be bioengineered (increased) through effective process control to improve removal rates in aerobic conditions (Confer and Logan, 1998; Hassard et al., 2016; Hassard et al., 2018).

Despite the relative importance of EEA for biological processes and wastewater modelling purposes, understanding the process or biochemical factors which determine the expression, regulation and activity of EEAs in wastewater treatment remains poorly understood (Goel et al., 1997; Truu et al., 2009). Direct hydrolysis of slowly biodegradable substrates, elevated utilisation of storage compounds in the bacteria and removal of particulates in the biofilm itself have been suggested to improve performance in biological processes (van Loosdrecht et al., 1997; Goel et al., 1997; Goel et al., 1998; Goel et al., 1999). However, the reaction rates of these processes can take longer than the typical residence time within roughing biofilm reactors that can be as low as 5 min. Roughing reactors are very highly loaded and have short hydraulic retention time (HRT) with a low % removal but can result in significant performance enhancements when incorporated with other secondary treatment processes which incorporate a hybrid solids feed (Daigger and Boltz, 2011; Daigger et al., 1993; WPCF, 1988).

Incorporating a solids feed of active biological solids into a biofilm reactor in a hybrid configuration could permit elevated OLRs and increase removal efficiencies. Previous studies have operated hybrid reactors such as roughing trickling filter (TF)/activated sludge plant (ASP) (Daigger and Boltz, 2011) or roughing TF/TF (Daigger et al., 1993). These systems permitted the pre-treatment of wastewater prior to existing secondary treatment assets at higher OLRs than normally permissible. The return of settled sludge to the proximal end of a biological processes is thought to improve treatment efficacy through enhanced bacterial contact, elevated suspended solids concentration and elevated EEA (Daigger and Boltz, 2011; Hassard et al., 2015). Recycling of solid material also results recycling of some of the organic polymers which suggest organics received several 'contacts' with the biofilm. However, these potential benefits are balanced through the reduction in HRT, which inevitably occurs with higher organic OLRs and solids loading rates (SLRs). This reduces the time for the biological degradation of organic polymers. In addition, this could result in washout or inactivation of microbial community and extracellular enzymes in suspended growth systems. You et al. (2003) found that hybrid processes allow treatment at greater OLRs, nitrification at lower SRT and increased resilience to nitrification performance upsets, possibly due to the solids recycle. Solids could allow greater volumetric removal than single pass systems without significant extra aeration costs (Hassard et al., 2015). It is hypothesised that active bacterial solids can contribute to elevated EEA and therefore better performance compared to conventional biological processes (not membrane processes) which have been observed previously (Hassard et al., 2016). Recycling of 'active' biological solids increases the maximum removal rates which can be achieved in hybrid biofilm systems. Further, recycling of active solids increases the EEA in the biofilm through either biostimulation and/or bioaugmentation processes. To elucidate this, the impact of different potential biosolids types including final effluent (FE), humus solids (HS) and recycle activated sludge (RAS) on the performance of the biofilm microbial extracellular enzyme activity in hybrid rotating biofilm reactors (RBRs) was assessed. To test these hypotheses under representative conditions of roughing biological processes, controlled organic overloading was performed with 6 pilot scale RBRs to assess the impact of high OLRs and SLRs on the reactor performance and EEA surrogates. Specifically, we proposed that acclimation to substrate limited reactor concentrations caused by competition between biofilm and suspended bacteria would (1) lead to elevated reactor removal rates and (2) result in a transient change to the reactor biological function. Ultimately, this research aimed at explaining why some biofilm exhibit significant extracellular degradation of wastewater polymers, and others do not. To our knowledge, this is the first study to report the biodegradation potential and kinetics of EEA substrates in an active microbial biofilm receiving different solids recycle(s).

2. Materials and methods

2.1. Pilot scale studies at varying OLR and SLR using real wastewater

Six identical RBRs were situated at Cranfield University Wastewater Treatment Works (WWTW); each consisted of a plastic vessel and a single rotating shaft with permeable plastic frames containing PVC-derived mesh ($n = 2$, d = 0.02 m, thickness = 0.05 m, porosity = 95%, submergence = 40%, wetted reactor volume = 3 L). The RBRs were operated at a constant tip speed (0.08 ms^{-1}) and fed with real settled sewage and operated for a period of 9 months. Solids were obtained from the final clarifier from a full-scale municipal WWTW, which was situated after secondary treatment with TF treating a population equivalent of 4000 PE. The FE was obtained from final effluent chamber prior to the environmental discharge point of the same WWTW. The HS and FE were fed into the RBRs by peristaltic pumps from 400 L holding tanks (T425NA12GH, Tanks-Direct, UK) which were refreshed daily; the solids were mechanically stirred to prevent settlement (Fig. 1), wastewater constituents were monitored daily. The RAS was provided by a pilot scale activated sludge process (ASP) characterised by Petrie et al. (2014) operated at a sludge retention time (SRT) of 20 days and a HRT of 16 h. The SRT was controlled through daily wasting of solids and calibration based on the mixed liquor content of the suspended phase. The RAS was pumped from the final settlement tank of the pilot scale ASP at the same flowrate as FE and HS (Fig. 1). All solids were taken from reactors which were treating real settled municipal wastewater which was from the same origin as that treated by the RBRs. Different total OLRs and SLRs were applied to each FE, HS and RAS reactor to understand the impact of OLR, SLR and solids type on the performance and microbial EEA of the biofilm reactor. Different nominal OLRs were applied to each RBRs at ~72, 152, 351, 546 g sCOD m^{-2} d^{-1} corresponding to 50, 100, 200, 400 L d^{-1} of settled

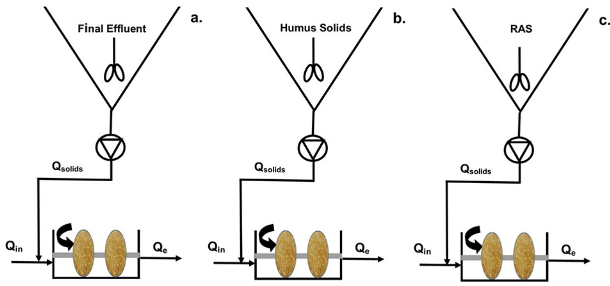


Fig. 1. Experimental setup for bench scale hybrid rotating biofilm reactors. With influent (Q_{in}), effluent (Q_e) and solids flows (Q_{solids}). Sample points were from influent, effluent and solids feed from each reactor. The final effluent and humus solids were mixed mechanically, whereas the RAS was from a reactor which was mixed through aeration, which was subsequently settled.

wastewater per RBR per day. These amounts are excluding any organic load from the solids feed. The OLRs were equivalent to a BOD_5 loading rates of 9.1, 15.7, 44.3 and 68.8 kg BOD_5 $m^{-3}d^{-1}$, respectively. Therefore, the hybrid RBRs operate between 1 and 8 fold higher OLR than conventional biological roughing processes such as TF and between 1 and 4 fold higher OLR than synthetic media filters (Metcalf and Eddy, 1979; WPCF, 1988). Two different solids recycle rates (50% and 100% of influent flow) were applied to each reactor (solids type) at each OLR treatment (which in total was 24 treatments). The HRT therefore decreased incrementally from 57.6 min at low OLR, low SLR to a minimum of 5.4 min at the very high OLR and high SLR condition (Table 1). It is acknowledged that there would be low % COD removal performance at this low HRT however substantial improvements in overall removal rate were envisaged. Temperature was recorded in the bulk fluid of RBR reactors, the settlement tank of the pilot scale activated sludge reactor and holding tanks for FE and HS (EL-WiFi-TP$^+$, Corintech, UK).

2.2. Wastewater analysis

Samples were collected from the influent and solids feeds at 09:00 ± 1 h, daily in triplicate. The effluent samples were collected at one HRT post influent sampling and also in triplicate. Wastewater was analysed using proprietary cell test kits (Hach-Lange, Germany) for total chemical oxygen demand (COD), total nitrogen (TN), ammonia-nitrogen (NH_4-N), nitrite nitrogen (NO_2-N) and nitrate-nitrogen (NO_3-N) using a Hach DR 2800 spectrophotometer (Hach-Lange, Germany). Biochemical oxygen demand, mixed liquor suspended solids (MLSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA-AWWA-WEF, 2012) as outlined in Hassard et al., 2018.

Table 1
study conditions of hybrid RBRs operated under incrementally increasing OLR and SLR. The SLR was different between each reactor type and controlled by flow rate. Key process parameters such as total flow to RBR and HRT were constant between reactor type.

Organic Loading	Recycle flow as ratio of influent	Influent flow rate (L d^{-1})	Solids flow rate (L d^{-1})	Total flow to RBR (L d^{-1})	RBR HRT (mins)
Low	0.5	50	25	75	57.6
	1	50	50	100	43.2
Medium	0.5	100	50	150	28.8
	1	100	100	200	21.6
High	0.5	200	100	300	14.4
	1	200	200	400	10.8
Very high	0.5	400	200	600	7.2
	1	400	400	800	5.4

The removal efficiency (e.g. bulk organics, NH_4-N) was calculated after accounting for influent flow, solids flow and respective concentrations after Eq. (1):

$$\% = (S_i + (R * S_{solids}) - S_e)/(S_i + (R * S_{solids})) * 100 \quad (1)$$

where S_i = the influent substrate concentration, R = proportion of flow is solids, S_{solids} = the substrate concentration in the solids flow, S_e = effluent substrate concentration.

In Eq. (1) the solids flow is similar to a return flow or recycle in conventional secondary treatment processes, such as in an ASP.

The substrate loading rate (X [either organics, NH_4-N, NO_x-N]-LR) was calculated taking into account concentrations and flows of influent and solids feeds after Eq. (2):

$$X{-}LR_{nominal} = (S_i * Q_i) + (S_{solids} * Q_r)/SA_{nominal} \quad (2)$$

where Q_i = influent flow rate, Q_r = solids flow rate, $SA_{nominal}$ = the nominal mesh surface area.

The removal rate for sCOD and NH_4-N was calculated based Eqs. (1) and (2).

The solids loading rate (SLR) was calculated taking into account solids flow after Eq. (3):

$$SLR_{nominal} = (TSS_{ret} * Q_r)/SA_{nominal} \quad (3)$$

where TSS_{ret} = total suspended of solids flow.

The nitrogen removal efficiency (TN) was calculated considering the influent and recycle NO_x concentrations (NO_2-N, NO_3-N) and accounting for internal nitrification after a simplified mass balance Eq. (4):

$$TN = ((NO_x{-}N_i + NO_x{-}N_s + NO_x{-}N_n) - (NO_x{-}N)_e/(NO_x{-}N_i + NO_x{-}N_s + NO_x{-}N_n) * 100 \quad (4)$$

where: NO_x-N_i = Influent NO_x concentration, NO_x-N_s = Solids NO_x-N concentration, NO_x-N_n = internal NO_x-N generation by nitrification, NO_x-N_e = Effluent NO_x-N concentration.

The wastewater composition was similar between reactors and did not differ significantly between OLRs treatments ($p < .05$). The ambient air in the test facility was heated to 17.5 ± 2.1, 18.8 ± 1.8, 17.2 ± 0.9 and 16.8 ± 0.8 °C for low, medium, high and very high OLRs to ensure the temperature was similar between commissioning, acclimation and steady state (sampling phase) for the duration of the study.

2.3. Extracellular enzyme activity assays

The biofilm was harvested from mesh media (Hassard et al., 2016) and mixed liquor suspended solids (MLSS) was taken from influent and effluent sampling points (influent, effluent and solids). Samples were subjected to identical pre-treatment. Appropriate buffer (different for each substrate) and methanol (10% v:v) was used to disperse biofilm (Lunau et al., 2005). Biomass was then disrupted mechanically using a homogeniser (T50 Ultra-turrax, IKA, Germany) for 1 min at 6000 rpm, to reduce mass transfer limitation (Hassard et al., 2014). Finally, the biomass was handled by pipetting (Finntip™ Wide Orifice Pipette Tips, Thermofisher, UK). The biofilm total solids concentration was measured after the method in Regmi et al. (2011).

Enzyme assays were undertaken after method outlined in Hassard et al. (2018). The initial rate of enzyme substrate reaction was used to calculate the EEA. The V_0 is defined as the initial velocity of the enzyme/substrate reaction. The V_{max} is defined as the maximum enzyme activity achievable in a dynamic system, where substrate (S) concentration does not limit the reaction rate which can be achieved. The K_m is the apparent Michaelis-Menten constant and is defined as the substrate concentration at half the V_{max} (Chen et al., 2010). First, the Michaelis-Menten Eq. (5) was solved using a non-linear least squares method for kinetic parameter estimation (V_{max}, K_m); then the standard error of mean and significance of model fit were calculated using a Hessian matrix and *t*-test respectively according to Hassard et al. (2018). The specific enzymatic activity was quoted as the maximum rate per gram of volatile suspended or immobilised solids which was calculated after Eq. (6):

$$V_0 = (V_{max} * S_m)/(K_m + S_m) \quad (5)$$

$$\text{Maximum Specific EEA} = V_{max}/VS_b \quad (6)$$

where VS_b is the volatile solids of the biofilm

2.4. Statistical analysis

Separate hierarchical multiple linear regression analysis (MRA) was undertaken to understand the impact of independent variables (Solids type, OLR, NH_4-N-LR, NO_3-N-LR, oxygen concentration, redox potential, pH, biofilm specific EEA and K_m on the variability in dependent variables (sCOD removal rate, NH_4-N removal rate, NO_3-N removal rate). In all cases the assumptions of MRA were met and standardised regression coefficients (β coefficient) permitted comparison of the impact of the dependent variables on the removal rates on the same scale (Germain et al., 2005). Correlation analysis between each independent variable showed that OLR, NH_4-N-LR, NO_3-N-LR were highly correlated with each other ($R^2 > 0.75$) therefore only bulk organics 'OLR' was used represent each substrate 'loading' in each substrate specific MRA model. In addition to MRA, a separate three-way ANOVA was used to test for an interaction effect between OLR, SLR and solids type. The was no significant three-way interaction identified, therefore two-way main effects were calculated for each factor and pairwise comparisons were used to compare differences. The assumptions of ANOVA were met and the difference in the ANOVA was deemed significant at $p < .05$. A multivariate distant based linear model (DistLM) were constructed to assess the effect of different reactor process conditions on the reactor performance and EEA (Anderson et al., 2008).

3. Results

3.1. Operating parameters

The influent municipal settled sewage had average concentrations of 630 ± 505 mg L^{-1} tCOD, 122 ± 22 mg L^{-1} sCOD, 31 ± 6 mg L^{-1} NH_4-N and 331 ± 203 mg L^{-1} TSS, respectively during the sampling campaign. The reactor temperature in the RBRs and ASP did not change by >12% between day and night. The total OLRs increased as expected based on the set influent flow rates and were not significantly different between solids types (Tables 1 and 2). The influent sCOD and NH_4-N concentration did not exceed ±20% standard deviation between different solids type and OLR/SLR treatments. The average concentration of solids in the recycle feed was 49, 1240 and 1819 mg L^{-1} for the FE, HS and RAS respectively. These solids concentrations were similar during the experimental period. The NO_3-N concentration in the influent remained low (<2.5 mg L^{-1}) throughout the study whilst the recycle NO_3-N was 28.9 ± 6.6, 29.3 ± 5.8 and 24.9 ± 5.2 on average for FE, HS and RAS respectively i.e. no statistical difference between the NO_3-N concentration in the biosolids feeds. The NO_3-N-LR increased as expected based on recycle flows and was similar for each solids type (Table 2) and the low SLR had ~50% of the NO_3-N-LR compared to the high SLR (Table 2). The very high OLR treatment was the exception as the NO_3-N concentration in the RAS recycle feed decreased by 67% to 8.2 ± 4.3 mg L^{-1}, due to denitrification in the final clarifier of the ASP at these very high recycle rates. The reactor DO decreased from ~5 mg L^{-1} to 1.4–1.9 mg L^{-1} at low and very high OLR respectively, for both FE and HS reactors due to overloading. The RAS reactor DO was between 1 and 1.8 mg L^{-1} at the low OLR and decreased to <0.7 mg L^{-1} at very high OLR (Table 2). In general, the RBR reactor DO decreased with higher OLRs and SLRs (Tables 1 and 2) principally due to lower HRT for oxygenation and greater oxidation of bulk organics.

3.2. Impact of OLR and SLR on the performance of the rotating biofilm reactors

3.2.1. Organics removal performance

At low OLR, the RAS reactor had a removal rate of ~50 g sCOD m^{-2} d^{-1} twice that of FE or HS reactors (Fig. 2a). The sCOD removal rate increased in a pseudo-linear fashion from low to very high OLR to a maximum of 231 g sCOD m^{-2} d^{-1} for the RAS reactor attained at the low SLR treatment. This represented a ~6-fold increase on the performance of identical RBRs were operated without a solids feed (Hassard et al., 2014). This finding suggests that the solids feed/dilution of influent wastewater improved bulk organics removal despite reductions in HRT (Table 1). Despite overloading, more COD was removed per unit of reactor (Fig. 2a). In this study very high OLR/high SLR treatment reduced the sCOD removal rate by 17, 9 and 26% for FE, HS and RAS respectively compared to the treatment with equivalent OLR but lower SLR (Fig. 2a). This suggested the HRT threshold in terms of the removal rate performance had been reached. The importance of each parameter for the variability in sCOD performance (ranked from greatest to least) was OLR > Solids type > phosphatase EEA (sCOD model, R^2 = 52%, Table 3) with OLR accounting for the majority (82%) of the variability in sCOD removal rate.

Table 2
Operating conditions in hybrid RBRs operated with different solids loading rates (SLR) set at 50% ¦ and 100% of influent flow rate.

Organic loading→	Low			Medium			High			Very High		
Solids type →	Final effluent	Humus solids	Recycle activate sludge	Final effluent	Humus solids	Recycle activate sludge	Final effluent	Humus solids	Recycle activate sludge	Final effluent	Humus solids	Recycle activate sludge
Total OLR ($gm^{-2}\cdot d^{-1}$)	78 ¦ 88	78 ¦ 87	82 ¦ 95	180 ¦ 209	185 ¦ 218	181 ¦ 211	422 ¦ 442	396 ¦ 439	399 ¦ 446	670 ¦ 776*	648 ¦ 731*	683 ¦ 801*
% OLR from solids	13 ¦ 32	11 ¦ 19	17 ¦ 28	16 ¦ 27	18 ¦ 30	16 ¦ 28	14 ¦ 24	12 ¦ 21	12 ¦ 22	16 ¦ 27	13 ¦ 22	18 ¦ 29
SLR ($kg\cdot TSS\cdot m^{-2}$ d^{-1})	0.05 ¦ 0.09*	0.13 ¦ 0.27*	2.88 ¦ 5.76*	0.05 ¦ 0.11*	1.68 ¦ 3.37*	3.75 ¦ 7.49*	0.08 ¦ 0.16*	5.1¦ 10.2*	10.9 ¦ 21.9*	0.25¦0.5*	6.51¦13.02*	14.59¦29.18*
NH_4-N-LR ($gm^{-2}\cdot d^{-1}$)	17 ¦ 17	16 ¦ 16	17 ¦ 19	41 ¦ 41	41 ¦ 41	41 ¦ 41	84 ¦ 85	83 ¦ 84	84 ¦ 87	165 ¦ 166	164 ¦ 164	191 ¦ 219
% NH_4-N-LR from solids	1.0 ¦ 2.1	0.2 ¦ 0.4	0.6 ¦ 1.1	0.9 ¦ 0.8	0.3 ¦ 0.5	0.2 ¦ 0.3	2.3 ¦ 4.4	1.3 ¦ 2.5	2.7 ¦ 4.9	0.9 ¦ 1.8	0.2 ¦ 0.4	12.3 ¦ 21.1
NO_3-N-LR ($gm^{-2}\cdot d^{-1}$)	7 ¦ 14*	9 ¦ 17*	6 ¦ 12*	19 ¦ 37*	21 ¦ 41*	46 ¦ 64*	46 ¦ 89*	42 ¦ 81*	33 ¦ 64*	78 ¦ 153*	75 ¦ 148*	27 ¦ 50*
Reactor oxygen concentration ($mg\cdot L^{-1}$)	5.2 ¦ 4.0	5.6 ¦ 4.6	1.8 ¦ 1	3.6 ¦ 2.1	3.7 ¦ 3.1	3.1 ¦ 2.4	1.3 ¦ 1.2	2.6 ¦ 2.1	1.9 ¦ 1.9	1.5 ¦ 1.7	1.4 ¦ 1.9	0.8 ¦ 0.6
Reactor redox (mV)	72 ¦ 66	70 ¦ 63	68 ¦ 92*	56 ¦ 39*	43 ¦ 40	43 ¦ 42	42 ¦ 23	29 ¦ 22	14 ¦ 12	25 ¦ 17	20 ¦ 16	14 ¦ 6*
Reactor pH (log_{10} [H_+])	7.6 ¦ 7.3	7.6 ¦ 7.7	7.5 ¦ 7.5	7.5 ¦ 7.7	7.4 ¦ 7.4	7.4 ¦ 7.5	7.6 ¦ 7.7	7.7 ¦ 7.7	7.6 ¦ 7.6	7.7 ¦ 7.7	7.7 ¦ 7.4	7.7 ¦ 7.8

(*) = difference between solids recycle ratio of 0.5 & 1 significant ($p < .05$). BOD loading rates are 9.1 (low), 15.7 (medium), 44.3 (high) and 68.8 (very high) $kg\cdot m^{-3}$ d^{-1}.

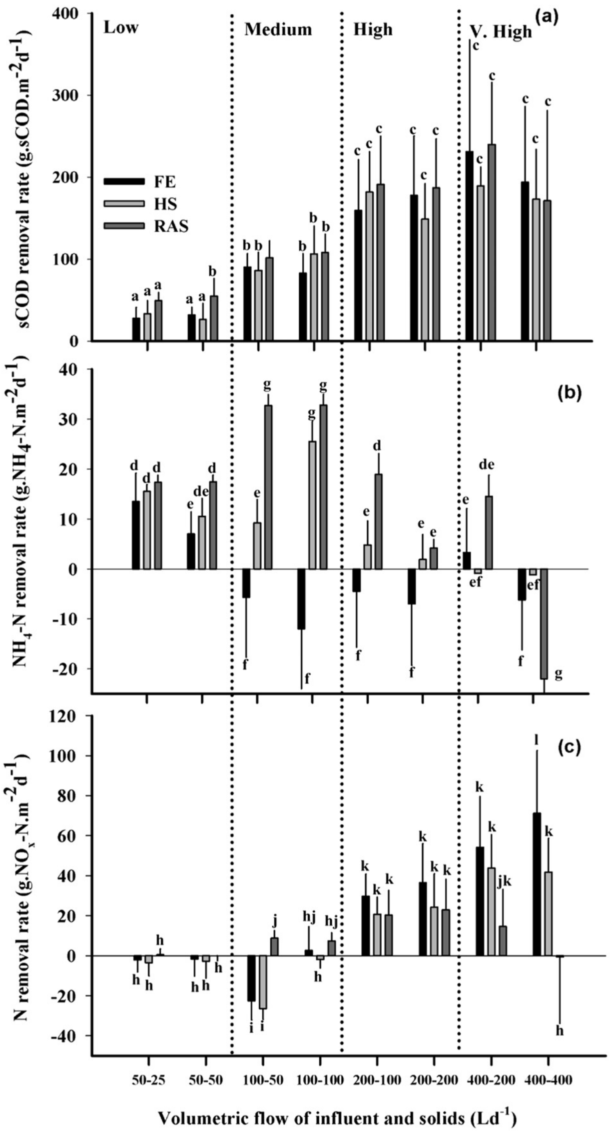


Fig. 2. Performance of hybrid rotating biofilm reactors (RBRs) for (a) soluble chemical oxygen demand (sCOD) removal (b) ammonia removal (NH_4-N) (c) nitrogen removal (NO_3-N) rates when the RBRs were operated at 50, 100, 200,400 Ld^{-1} influent flow rate. Solids recycles were: final effluent (FE), humus solids (HS), recycle activated sludge (RAS) which were fed into the reactor at 50% and 100% of the influent flow rates which were applied (x-axis). The data on each y-axis represent 6 independent reactors experiments with averages ± standard deviation of 15 replicate measurements at each OLR/SLR treatment over a 1-month operating period. Like letters indicate no significant differences ($p > .05$) when between treatments (pairwise comparisons).

3.2.2. Ammonia removal performance

The FE reactor removed NH_4-N at the low OLR/low SLR whilst at the high SLR the removal decreased by 48% ($p < .05$) but there is still some removal at high SLR (Fig. 2 b). The HS reactor performed in a similar way for the low OLR, attaining 91 and 35% NH_4-N removal efficiency at the low and high SLR respectively (Fig. 2b). In contrast the RAS reactor had 94 and 78% NH_4-N removal rate at low and high SLRs respectively. Thus the reductions in HRT had less of an impact on observed NH_4-N removal when RAS was utilised as solids feed, probably due to augmentation of extra nitrifying bacterial populations to the biofilm reactor within the RAS flocs. At the medium OLR, the RAS reactor outperformed both the FE and HS reactors ($p < .05$) with a maximum NH_4-N removal rate of ~31 g·NH_4-N m^{-2} d^{-1} achieved at both the low and high SLR. In this study, the NH_4-N removal rate reduced by 48 and 92% (low and high SLR) for HS and by 41 and 89% (low and high SLR) in the RAS (Fig. 2b). This suggested that bioaugmentation of active nitrifying

Table 3
Multiple linear regression (MLR) output β coefficients. The dependent variable for each model was the removal of sCOD, NH_4-N and NO_3-N. The independent variables were Solids type, OLR, SLR, NH_4-N-LR, NO_3-N-LR, Reactor chemistry (pH, O_2, redox), potential), recycle chemistry (pH, O_2 and redox potential), amino-peptidase specific EEA, phosphatase specific EEA, amino-peptidase K_m and phosphatase K_m.

Model (dependent variable →) independent variables↓,	β coefficient sCOD[c] $R^2 = 56\%$	NH_4^b $R^2 = 33\%$	NO_3^b $R^2 = 54\%$
Solids type	**0.19[a]**	**0.44[a]**	**0.14[c]**
OLR	**0.82[a]**	–	–
SLR	**−0.21[a]**	−0.18[d]	**−0.24[a]**
NH_4-N-LR	–	−0.08[d]	–
NO_3-N-LR	–	–	**0.66[a]**
Reactor pH	–	**−0.17[a]**	–
Recycle pH	–	−0.04[d]	–
Reactor O_2	–	–	**−0.15[b]**
Recycle O_2	–	–	0.06[d]
Reactor redox potential	–	**−0.13[c]**	–
Recycle redox potential	–	**0.31[a]**	–
Biofilm amino-peptidase specific EEA	0.03[d]	0.02[d]	–
Biofilm phosphatase specific EEA	**0.16[c]**	−0.05[d]	–
Biofilm amino-peptidase K_m	–	–	**0.12[c]**
Biofilm phosphatase K_m	–	–	−0.01[d]

Significance of MLR model a ≤ 0.001, b ≤ 0.01, c ≤ 0.05 d ≥ 0.05; (—) = variable excluded from regression model, as not correlated with performance. Bold indicates variable that adds significant value to the multiple linear regression model.

bacterial solids (e.g. HS or RAS) was critical to maintain high NH_4-N removal rates at OLRs which were in excess of 80 g sCOD m^{-2} d^{-1}, compared to the FE reactor (which was representative of systems with similar HRT but limited active solids within feed). The reactor conditions which were most important for the variability in NH_4-N removal was solids type > recycle redox potential > reactor pH > reactor redox potential (NH_4-N model, $R^2 = 0.33$, Table 3). The solids type and recycle redox potential accounted for 44 and 31%, respectively of the variability in the NH_4-N removal performance. Both augmentation of active nitrifying solids and aerobic conditions were required for elevated NH_4-N removal performance (autotrophic nitrification + and assimilation) (Fig. 2b, Table 3).

3.2.3. Nitrogen removal performance

Overall, the hybrid RBRs did not remove NO_x-N at low OLR, as the HS and FE reactors had high effluent NO_x-N which was 19.2–26.2 mg L^{-1}. The RAS reactor was the exception, as it had lower effluent NO_x-N which was 9 and 7 g m^{-2} d^{-1} for low and high SLR, respectively at medium OLR. This suggested that at medium OLR, there was the onset of simultaneous nitrification/denitrification or assimilation of nitrogen within the biofilm (Fig. 2b and c), which was confirmed by low NO_x-N in the influent, good NH_4-N removal (81%) and slight reduction in the NO_x-N in the effluent. At the very high OLR, the NO_x-N removal rate for FE and HS increased to a maximum of 42 and 71 g NO_x-N m^{-2} d^{-1}, respectively with the FE reactor outperforming the HS and RAS reactors ($p < .05$) (Fig. 2c). The factors which governed the variability in NO_3-N removal rate were: NO_3-N-LR > SLR > reactor O_2 > Solids type > Biofilm amino-peptidase EEA (β-coefficients ranked in order from greatest to least). The maximum NO_x-N removal rate of the RAS reactor was 23 g m^{-2} d^{-1} at the high OLR and high SLR treatment, similar to the HS removal despite 21% lower NO_3-N-LR supplied to the RAS reactor, suggesting greater nitrogen removal efficiency (Fig. 2c, Table 2). The nitrogen removal performance of the RAS reactor was restricted by experimental restrictions notably NO_3-N-LR.

3.3. Microbial extracellular enzyme activity

The experimental data did not differ significantly from the Michaelis–Menten model for all V_{max} and K_m treatments (t-test between observed and expected, p < .05) which suggested this model was suitable. The maximum number of iterations to convergence for the EEA models was <2 in all cases. The achieved convergence tolerance was $<3 \times 10^{-6}$, which is below the accepted upper limit of 1×10^{-4}, suggesting low error accumulation and therefore model accuracy to achieve convergence (Sacchi Landriani et al., 1983).

The amino-peptidase EEA was minimal and ranged from 9 to 15 μmol g VS^{-1} min^{-1} between low OLR (low and high SLR) and medium OLR/low SLR for all reactors. Amino-peptidase EEA increased by 76, 35, and 63% for FE, HS and RAS, respectively with medium/high SLR compared to the low SLR (Fig. 3a, $p < .05$). At high OLR/low SLR the amino-peptidase EEA increased by ~3 and ~5 fold for both FE and RAS reactors ($p > .05$). The EEA reached a maximum of 122 and 115 μmol g VS^{-1} min^{-1} at high OLR/low SLR for FE and RAS reactors, respectively. This was not found in the HS reactor as amino-peptidase EEA increased by ~30%. The FE reactor amino-peptidase EEA decreased to ~40 μmol g VS^{-1} min^{-1} for at OLRs > 400 g sCOD m^{-2} d^{-1} and high SLR (Fig. 2a). The EEA in HS and RAS reactors declined rapidly to minimal at HRTs < 14 min (Fig. 3a and b; Table 1). The trend was similar for phosphatase EEA which increased with OLR to a maximum of 55.1 μmol g VS^{-1} min^{-1} at high OLR/low SLR treatment for the RAS reactor, however the EEA was ~50% of amino-peptidase and activity in FE and HS reactors (Fig. 3b).

The amino-peptidase K_m increased with OLR suggesting that at higher OLR the biofilm reaches V_{max} more slowly. The RAS reactor had similar K_m of 789 and 937 μM despite EEA of 86 and 116 μmol g VS^{-1-} min^{-1} at the high OLR for low and high SLR respectively. At the very high OLR the FE reactor had a K_m which was 46 and 32% greater than the RAS reactor at low and high SLR respectively (Fig. 3 c). The phosphatase K_m was similar between reactors under most conditions studied although a marked decline in K_m of 85 and 49% between high and very high OLRs for the RAS reactor at low and high SLR respectively, this trend was reflected in all reactors (Fig. 3 d). DISTLM revealed that 90% of the variability in performance of hybrid RBRs was governed by the physico-chemical variables with most 56% being governed by the OLR, SLR and solids type (Fig. 4).

4. Discussion

In this study we demonstrated that the addition of biosolids feed to a RBR increases the removal rates of COD and NH_4-N at low OLR. Furthermore, at very high OLR addition of the biosolids feed increased the removal rates NO_x-N by 66% for FE and 39% for HS compared to RAS. Further to this, the increase of the biosolids feed delayed the reduction in removal efficiency of organics and inorganics observed in high OLR biofilm processes. In other studies, the biosolids feed was shown to also dilute the influent wastewater, resulting in elevated reactor dissolved oxygen (DO) and a reduction in the presence of filamentous microbiota (Ayoub et al., 2004; de Kreuk et al., 2010). Together these factors result in higher removal rates in hybrid reactors despite similar reactor volumes to single pass conventional biofilm processes (Hassard et al., 2016). In granular systems, de Kreuk et al. (2010) showed that reducing substrate gradients between the water phase and the biofilm reduced the competitive advantage of filamentous microbiota, which, are typically found in biofilm processes operated at very high OLR (Ayoub et al., 2004). Another factor important for the removal of organic polymers from wastewater using biofilms is that of effective surface area for bacterial contact (Daigger et al., 1993). Addition of solids into the biofilm reactor increases the surface area within the suspended phase of the reactor which firstly increases the area for adsorption and secondly increases the substrate contact with key extracellular enzymes and bacteria (Confer and Logan, 1998). In this study, the lower sCOD removals seen at the very high OLR/high SLR treatments (Fig. 4 b) are likely due to a combination of DO limitation (reactor DO

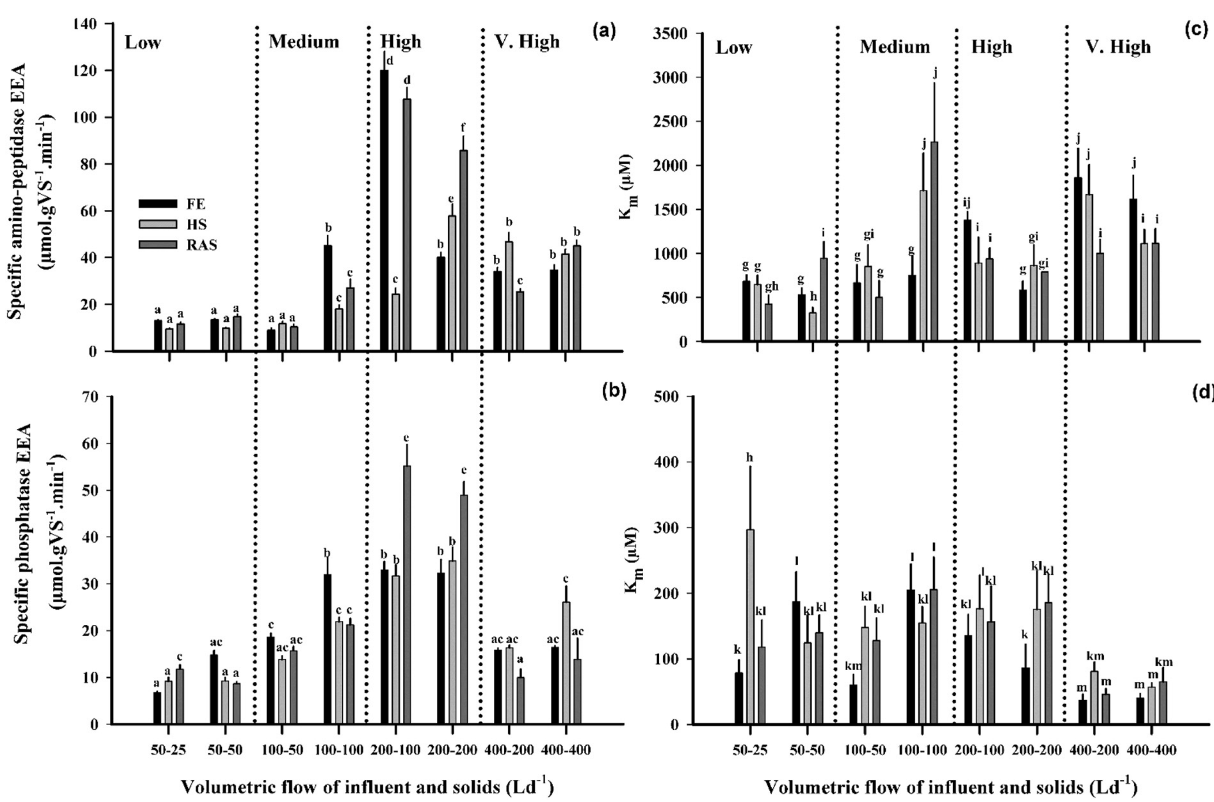
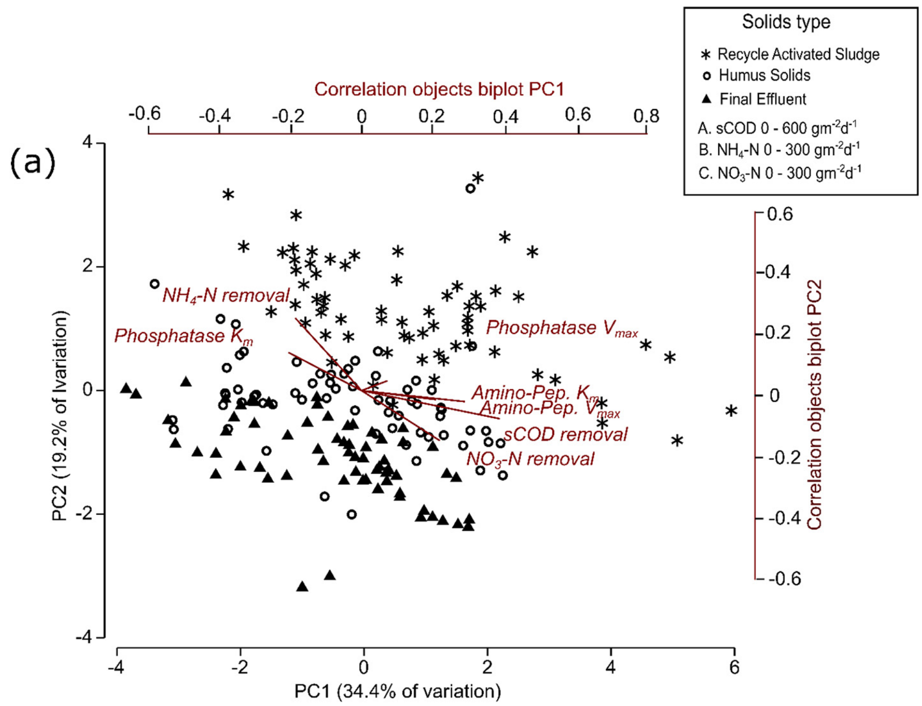


Fig. 3. Microbial extracellular enzyme activity (EEA) of the biofilm from hybrid rotating biofilm reactors (a) Specific amino-peptidase EEA (b) Specific phosphatase EEA(c) Amino-peptidase K_m (d) Phosphatase K_m. Solids recycles were: final effluent (FE), humus solids (HS), recycle activated sludge (RAS) at 50% and 100% of influent flow which were applied to each RBR. Bars represent data from 6 independent reactors (low and high - SLR, FE, HS, RAS), displayed as averages and sd. of triplicate assays on 6 artificial substrate concentrations. Each bar represents the average EEA over a 1-month operating period. Like letters indicate no significant differences ($p > .05$) between treatments (pairwise comparisons).



(b)

Sequential test Variable	Cumulative R^2	Pseudo-F	P	res.df
OLR	0.31	68.5	< 0.001	149
SLR	0.44	32.1	< 0.001	148
Solids type	0.56	41.4	< 0.001	147
Reactor pH	0.63	25.8	< 0.001	146
Reactor DO	0.72	44.9	< 0.001	145
Reactor redox potential	0.84	113.5	< 0.001	144
Recycle redox potential	0.90	88.4	0.05	143

Fig. 4. PC1 versus PC2 scores for reactor variables which were: recycle dissolved oxygen, reactor dissolved oxygen, redox potential of recycle, redox potential of reactor, reactor pH, solids type, organic loading rate and solids loading rate (most relevant determined by BEST analysis). The reactor performance criterion were: sCOD, NH_4-N and NO_3-N removal performance and extracellular enzyme activity (EEA) which were from hybrid rotating biofilm reactors using final effluent (FE), humus solids (HS), and recycle activated sludge (RAS) as a recycle feed (a) a Pearson's correlation (−1 to 1) is overlain on the principal component plot in the form of a vector plot to visualise the correlation between reactor performance and reactor conditions (b) Distance Based Linear Model sequential test output showing the influent of reactor physico-chemical parameters on the reactor performance (EEA and the sCOD, NH_4-N, NO_3-N removal rates).

typically < 1 mg L^{-1}) and HRTs of ~5 min, which, is less than the established generation time of most wastewater bacteria.

Another mechanism explaining low EEA under high OLR is the inhibition and eventual decay of aerobic protozoa under anaerobic conditions (Hauduc et al., 2013). At high OLRs the viability of aerobic bacteria can also be reduced, despite greater biomass concentrations (Hassard et al., 2014, 2015). Feast-famine conditions, such as those experienced in the hybrid RBR, could encourage the uptake and utilisation of storage products within the bacteria of the biofilm. Recycling bacteria from the end of a WWTW (famine) to the front of the WWTW (feast) analogous to ASP and RBR in this study can promote a reduction in degradable sCOD through adsorption, EEA and subsequent storage and utilisation (van Loosdrecht et al., 1997)

High NH_4-N removal rates (>78%) were observed in the RAS solids RBRs at higher OLRs than previously established thresholds of 15 g·BOD_5·$m^{-2}d^{-1}$ for some rotating biological contactor reactors (Hassard et al., 2015), and 35 g sCOD m^{-2} d^{-1} for mesh media reactors without solids feed (Hassard et al., 2014). In this study OLRs > 50 g sCOD m^{-2} d^{-1} addition of FE (minimal solids) was not sufficient to maintain NH_4-N removal efficiencies possibly through competition with heterotrophic bacteria (Wijeyekoon et al., 2004). In contrast HS and RAS reactors achieve removal rates of >10 and >30 g NH_4-N m^{-2} d^{-1} at sCOD loadings rates of ~180 g^{-2} d^{-1} (BOD_5 loading rate of ~260 g m^{-2} d^{-1}). You et al. (2003) found that hybrid processes allow treatment at greater OLRs, NH_4-N removal at lower SRT and increased resilience to performance upsets compared to conventional suspended growth systems. Comparison between the data presented in this study and other systems suggests hybrid RBRs offer elevated EEA compared to UASB, plug-flow digesters, RBR and ASP (Table 4). Although lower EEAs are reported for hybrid RBRs compared to some MBRs although most MBR papers present cumulative as opposed to instantaneous EEA (Table 4). It was suggested that the greater nitrifiers abundance or activity in hybrid systems governed the reactor performance for NH_4-N removal. The origin of the solids could influence nitrifiers abundance or activity, as the RAS came from an ASP whereby growth rate is controlled by sludge wasting. In contrast the bacterial solids in the HS are from a TF, which will have a variable sludge age and therefore growth rate (Bryers, 2000). In addition, most of the bacteria from a HS are sloughed or eroded (assumed inactive or decaying) which could explain their lower impact to NH_4-N performance. This would be particularly important during periods of TF die-off (Daigger and Boltz, 2011). Satoh et al. (2003) demonstrated that augmentation of nitrifiers into a RBC biofilm resulted in quicker start-up and elevated removal rates through greater nitrifiers density during the reactor start-up phase. Therefore, the HS and RAS RBR reactors could have had more active nitrifiers operating within the biofilm compared to the FE reactor.

Table 4
Comparison of EEA between different wastewater reactors at different OLR. Maximum enzyme activity reported used in all cases.

Sample source	Surrogate EEA compound used (concentration range)	Organic loading rate (units)	V_{max} or total enzyme activity.	Apparent K_m (μM)	Wastewater treated	Reference
Activated sludge model reactors	Amino-peptidase*†‡	/	38.4 μM min^{-1} 45.2 μM min^{-1} 19.7 μM min^{-1}	2697.2 2073.5 14,319	Synthetic dairy Synthetic municipal Synthetic petroleum	Li and Chróst, 2006
Membrane bioreactor	Alkaline Phosphatase§	1.7 kg·BOD·m^{-3}·d^{-1}	10,100 μM gVSS^{-1}·min^{-1}	/	Real municipal	Molina-Muñoz et al., 2010
Anaerobic digestion (plug flow)	Alkaline Phosphatase§‖	12.2 kg·COD·m^{-3}·d^{-1}	0.7 μM gVSS^{-1}·min^{-1}	/	Real distillery	Zhenglan et al., 1990
Upflow anaerobic sludge blanket digester	Alkaline Phosphatase§‖	32.6 kg·COD·m^{-3}·d^{-1}	2.1 μM gVSS^{-1}·min^{-1}	/	wastewater	
Constructed wetland	Alkaline Phosphatase§‖	/	11.5 μM·g^{-1} min^{-1}	/	Real municipal	Yuan et al., 2016
RBR	Amino-peptidase*†‡	11 kg·tCOD·m^{-3}·d^{-1}	26 μM·gTS^{-1}·min^{-1}	2100	Real municipal	Hassard et al., 2018
RBR	Alkaline Phosphatase*†‡	11 kg·tCOD·m^{-3}·d^{-1}	5 μM·gTS^{-1}·min^{-1}	498	Real municipal	Hassard et al., 2018
Hybrid RBR using RAS	Amino-peptidase	44 kg·BOD·m^{-3}·d^{-1}	110 μM·gVS^{-1}·min^{-1}	998.7	Real municipal	This study
Hybrid RBR using FE	Amino-peptidase	44 kg·BOD·m^{-3}·d^{-1}	122 μM·gVS^{-1}·min^{-1}	1400.2	Real municipal	This study
Hybrid RBR using HS	Amino-peptidase	44 kg·BOD·m^{-3}·d^{-1}	23 μM·gVS^{-1}·min^{-1}	800.4	Real municipal	This study

* = Same substrate concentration as this study.
† = kinetic parameters calculated from least squares regression.
‡ = Same buffer and pH as this study.
/ = data not presented or available.
§ = Kinetic parameter not presented total enzyme activity reported.
‖ = enzyme activity estimated from graph.

Nogueira et al. (2002) showed that shorter HRT mixed community nitrifying biofilms were more resilient to high OLR conditions than longer HRT biofilms, suggesting hybrid systems offer resilient, high rate nitrification.

The phosphatase K_m negatively correlated with nitrogen removal performance, suggesting that the K_m for phosphatase increased at higher OLRs as V_{max} was approached more quickly (Lehninger et al., 2005). Hanhan et al. (2005) found a nitrogen removal rate of 2.06 g N m^{-2} d^{-1} in an RBC system with a HRT of ~30 mins. In this study nitrogen removal rates > 60 g NO_x m^{-2} d^{-1} was found in hybrid RBRs. High biofilm nitrogen removal rates at HRTs of ~5 min demonstrate pre-denitrification and the roughing potential of hybrid RBRs.

The relationship between EEA, reactor physiochemical conditions and performance has rarely been effectively quantified in biofilm processes but remains crucial for effective monitoring and modelling of these systems (Hauduc et al., 2013). Unsurprisingly, the performance (removal of constituents and EEA pre-treatment) was mostly dependent on key process conditions important for hybrid biofilm systems, the OLR, SLR and solids type. Data presented here suggests that the EEA and the removal of organics and NO_x-N increased with OLR and SLR in hybrid RBRs. Furthermore, the type of bacterial solids can influence the EEA of the biofilm component of the RBR. Of the biosolids types studied, the RAS and HS had similar impacts on the NH_4-N performance but the FE-RBR performed best for NO_x-N removal possibly due lower competition with suspended flocculated bacteria for substrates. This basis of this research could be utilised to optimise/upgrade other biofilm processes for higher rate treatments, as here it has been shown that high levels of nutrient removal are permissible irrespective of the concentration or loading of the hybrid solids and are dependent instead on NO_x-N loading.

5. Conclusions

The operational data demonstrated that the flexibility of hybrid systems for removal or a range of constituents from wastewater. Batch experiments using the same biofilm, showed that EEA peaked at high OLRs. Therefore, this is the first study to offer an explanation as to why some biofilm have higher EEA than others during wastewater treatment. Elevated SLR of active solids delayed reduction in organic and inorganic removal rates common in biological processes under high OLRs. Different RBR function was evidenced due to the onset of denitrification in reactors dosed with some solids but not others.

Declaration of competing interest

The authors have no competing interests to declare.

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